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MitoVitE limits paclitaxel-induced oxidative stress and mitochondrial damage in vitro, and paclitaxel-induced mechanical hypersensitivity in vivo.

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MitoVitE limits paclitaxel-induced oxidative stress and mitochondrial damage *in vitro*, and paclitaxel-induced mechanical hypersensitivity *in vivo*.

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Abstract

Background

Neuropathic pain is a common side effect of chemotherapy and although precise mechanisms are unclear, oxidative stress and mitochondrial damage are involved. We investigated whether the mitochondria targeted antioxidant, MitoVitE, provided better protection against paclitaxel-induced mitochondrial damage in dorsal root ganglion (DRG) cells than a non-targeted form of vitamin E, Trolox. We also determined whether MitoVitE, compared to duloxetine, could limit paclitaxel induced mechanical hypersensitivity in rats.

Methods

Mitochondrial function was measured in DRG cells exposed to paclitaxel with and without MitoVitE or Trolox. The effect of MitoVitE or Trolox on paclitaxel-induced cell killing in cancer cell lines was also determined. Rats received a cumulative dose of 8 mg kg⁻¹ paclitaxel plus either MitoVitE (2mg⁻¹ kg day⁻¹), duloxetine (10mg kg⁻¹ day⁻¹) or vehicle control daily throughout the model. Mechanical hind paw withdrawal thresholds were measured every 2 days.

Results

Paclitaxel caused loss of membrane potential in DRG cells. At 100µM paclitaxel median [range] change was 60.8 [44.0-77.5]%, p<0.0001, which was ameliorated by MitoVitE (86.3 [62.3-103.8]%) but not Trolox (46.3 [46.3-57.4]%). Similarly, loss of metabolic activity and glutathione induced by paclitaxel (both p<0.0001) was also reduced by MitoVitE but not Trolox. Cytotoxicity of paclitaxel was not affected by co-exposure of ovarian cancer cells to either MitoVitE or Trolox but was slightly less effective against breast cancer cells in the presence of Trolox. Mean (SD) areas under the curve of withdrawal thresholds at 6h after injection of treatment in rats given paclitaxel + control, or + MitoVitE (p<0.0001) or + duloxetine (p<0.0001) were 110.1 (5.0), 144.9 (10.2) and 155.9 (12.6) respectively.

Conclusion

Paclitaxel affected mitochondrial function and glutathione in DRG cells, which was abrogated by MitoVitE but not Trolox, without decreasing cancer cell cytotoxicity. In rats, paclitaxel

induced mechanical hypersensitivity was ameliorated by MitoVitE treatment to a similar extent as duloxetine. These data confirm mitochondria as a mechanistic target for paclitaxel-induced damage and suggest mitochondria targeted antioxidants as future therapeutic strategies.

MESH key words

Pain, Paclitaxel, Mitochondria

Running title

MitoVitE limits paclitaxel-induced damage and mechanical hypersensitivity

Peer Review

Chemotherapy induced peripheral neuropathy (CIPN) is a common and severe adverse effect of some commonly used chemotherapeutic drugs, including paclitaxel. The severity of symptoms can require a dose reduction, or even cessation of chemotherapy, impacting on survival. CIPN affects around 33% of patients, and can persist months or years beyond the cessation of treatment. It is characterized by numbness, paresthesia, and pain.^{1 2} Duloxetine is the only treatment which has been shown in a randomized clinical trial to be effective in some patients with CIPN³ and is recommended as a first line treatment for adults with this condition. However, long term management of CIPN is often inadequate and there is urgent need for a mechanism-derived novel treatment.⁴

Mitochondria produce most of the body's cellular energy via oxidative phosphorylation, producing reactive oxygen species (ROS) in the process. Although ROS are potentially toxic, they have essential roles in cell signalling and any damage is controlled by an interacting and highly regulated system of endogenous antioxidants. As well as being the main source of ROS, mitochondria are also a target for damage and when antioxidant defences are overwhelmed, oxidative stress can result in mitochondrial dysfunction and impairment of ATP production. Thus damage to mitochondria is caused primarily by ROS produced by the mitochondria themselves.⁵

Paclitaxel has been shown to cause oxidative stress and mitochondrial damage⁶⁻⁷ and accumulates in neuronal tissue. Neuronal cells are particularly sensitive to oxidative insults, and ROS have been implicated in many neurodegenerative processes, including Alzheimer's, Parkinson's, and Huntington's diseases, acute brain ischaemia, and excitotoxicity. Neuropathic pain as a result of paclitaxel therapy may be related to mitochondrial damage to neuronal cells⁸⁻⁹ and so antioxidants which are able to protect inside mitochondria may be useful. Antioxidants can be targeted selectively to mitochondria by conjugation to a lipophilic cation.¹⁰ MitoVitE consists of α -tocopherol attached to a triphenylphosphonium (TPP) cation, enabling its rapid uptake through the plasma and mitochondrial membranes and accumulation within mitochondria as a result of the large membrane potential (negative inside) across the mitochondrial inner membrane. MitoVitE accumulates in all major organs of mice and rats after oral, intraperitoneal (i.p.) or intravenous administration.¹¹ Trolox (6-hydroxy-2,5,7,8-tetra methylchroman-2-carboxylic acid) is a synthetic, water soluble cell-permeable derivative of vitamin E which accumulates in the cell cytosol and has potent antioxidant activity with direct scavenging activity against peroxy and alkoxyl radicals.¹²

A number of rodent models of CIPN have been developed, including a paclitaxel induced CIPN model. Behavioural changes develop over time, with related neurobiological changes demonstrated, including mitochondrial dysfunction.¹³

The aim of this study was to investigate the effects of two vitamin E-based antioxidants in an *in vitro* model of paclitaxel-induced oxidative stress and mitochondrial damage in a DRG cell line, and effects on mechanical hypersensitivity in a preclinical model of paclitaxel CIPN in rats.

Materials and methods

In vitro studies

An immortalized dorsal root ganglion (DRG) neuronal stem cell line (50B11) with nociceptive properties was used (a kind gift from Professor Ahmet Hoke, from Johns Hopkins School of Medicine, Baltimore, MA, USA). When differentiated, these cells extend neurites, express the capsaicin receptor transient receptor potential vanilloid family-1 (TRPV-1) and other receptors characteristic of small sensory neurones, generate action potentials when depolarized, and respond to capsaicin.¹⁴ Cells (used at passage 5-15) were grown to 70% confluence in neurobasal media devoid of phenol red (Invitrogen, Paisley, UK) supplemented with 10% v/v foetal calf serum, B27 (Invitrogen, Paisley, UK), 2% w/v glucose, 0.5mM L-glutamine, 50µg/ml gentamycin, 250µg/ml amphotericin-B and 5µg/ml ciprofloxacin, in a humidified incubator containing 5% CO₂ at 37°C. Cells were then treated with 75µM forskolin and allowed to differentiate into DRG for 24h. Neurite outgrowth normally started around 10 hours after addition of forskolin. After 24h exposure to forskolin, paclitaxel (0-100µM) was added, corresponding broadly to levels seen in the circulation during clinical use. However, paclitaxel accumulates in cells and tissues and so the concentration range was extended to allow for this. In addition some cells were also concurrently exposed to 1µM MitoVitE, 1µM Trolox or a relevant solvent control.

Acid phosphatase activity

Acid phosphatase activity was used to assess cell viability.¹⁵ Differentiated cells were grown in 96-well plates and treated as described above for 24h then washed twice with phosphate buffered saline (PBS). Acid phosphatase solution containing 0.1M sodium acetate, 1% v/v Triton X-100, 5mM p-nitrophenyl in distilled water (pH 5.0) was added to each well and cells were incubated in the dark for 1h at 37°C. Sodium hydroxide (0.25M) was added to stop the reaction and the absorbance measured using a spectrophotometer at 450nm.

Mitochondrial function

Mitochondrial membrane potential was analyzed in intact cells using the fluorescent probe JC-1 (5,5,6,6-tetrachloro-1,1,3,3-tetraethylbenzimidazolcarbocyanine iodide, Invitrogen, Paisley, UK). Briefly, after 24h treatments as described above, cells were washed with PBS and then incubated for 30 min with 7.5µM JC-1 in PBS at 37°C, in the dark. Following

incubation, cells were washed twice with PBS and the red/green fluorescence ratio was measured immediately.¹⁶ Results were corrected for cell viability.

Metabolic activity was assessed by measuring the rate of reduction of AlamarBlue™ in intact cells after 24h treatment as described above.¹⁷ AlamarBlue™ (Invitrogen) is a redox indicator that exhibits both fluorescent and colourimetric changes in response to changes in metabolic activity via oxidative metabolism. Briefly, following cell treatments, AlamarBlue™ was added to each well and fluorescence was measured every 15 min for 2h at 37°C. Metabolic activity was determined as the rate of change in fluorescence over time. Results were corrected for cell viability.

Glutathione (GSH)

Cellular GSH levels were measured as an indicator of oxidative stress. The lipophilic compound monochlorobrimane (MCB, Sigma, Dorset, UK) binds to GSH via the action of the enzyme glutathione-S-transferase. The fluorescence of the resulting MCB-GSH conjugate is proportional to cellular GSH concentration.¹⁸ Cells were treated for 24h as above, washed with PBS and incubated with 20µM MCB for 30 minutes at 37°C. GSH was analysed by measuring fluorescence at excitation/emission wavelengths of 340/520nm. Results were corrected for cell viability.

Cancer cell cytotoxicity

Since it is possible that antioxidants would either interfere with or enhance the ability of paclitaxel to kill cancer cells, we also conducted experiments using the breast adenocarcinoma-like oestrogen-sensitive cell line, MCF-7, and the ovarian carcinoma cell line A2780, to assess paclitaxel induced cell killing in the presence of the antioxidants. Cells were cultured in Dulbecco’s modification of Eagle’s medium (DMEM) supplemented with 4.5g/l glucose, 10% v/v foetal calf serum, 50µg/ml gentamycin and 250µg/ml amphotericin-B for 24h. Cell viability was measured using acid phosphatase activity as described above.

Animal model

All studies were carried out in accordance with Animals (Scientific Procedures) Act 1986, and within the confines of project and personal licences issued by the UK Home Office, following relevant aspects of the ARRIVE Guidelines. Male Sprague Dawley rats, bred in-house and weighing 250-300g (approximately 7 weeks old) were used. Rats were housed up to 6 per cage at 19-22°C, on a 12-hour light/dark cycle from 7am to 7pm. Animals had free access to food and drinking water. Animals were housed in the testing room throughout experiments and for at least three days prior to baseline sensitivity measures.

Rats were allocated by cage to receive either paclitaxel or its vehicle control (Cremophor/EL and ethanol 1:1 [v/v] and diluted with saline as for paclitaxel and hereafter referred to as Cremophor) with and without MitoVitE or duloxetine as positive comparator. Since treatment with paclitaxel may contaminate other animals via coprophagy, animals could not be individually randomised to treatment group. Rats received 4 x doses of 2mg kg^{-1} paclitaxel or Cremophor by i.p. injection every second day¹⁹ and also received either $2\text{mg kg}^{-1}\text{ day}^{-1}$ MitoVitE, $10\text{mg kg}^{-1}\text{ day}^{-1}$ duloxetine or equivalent vehicle control by daily i.p. injection starting 7d before paclitaxel administration, to allow steady state tissue concentrations to be reached and to allow assessment of any effects in naïve animals. The injection site was varied daily to minimise any local tissue damage.

For behavioural sensory testing, rats were acclimatised to testing apparatus in 2 x 20 min sessions on separate days prior to testing, and further habituated for 20 min immediately prior to testing on any given day. To ensure blinding of the tester to the treatment, rats from several cages were briefly combined in a single cage before testing commenced; group allocation was only confirmed by tail number after testing was complete. Mechanical withdrawal thresholds were measured using the up-down method using a series of von Frey filaments of varying weights to gauge the withdrawal threshold and thereby mechanical sensitivity.²⁰ Starting with a 2g filament, increasingly heavier filaments were applied to the plantar region of the hind paw until a paw withdrawal response was observed. Sequentially smaller filaments were then tested until no response was observed, then increased again until a response was observed. This approach was repeated until there were five measures after the initial response. The value of the final filament tested, and sequence of responses were then used to calculate the mechanical threshold using the equation devised by Chaplan *et al.*²⁰ Behavioural measures were undertaken 3, 6 and 9 h after the MitoVitE or duloxetine injection, every 2 d starting 3 d before paclitaxel administration and continuing until 14 d after initial paclitaxel administration.

Statistical analysis

For *in vitro* studies, 6 independent experiments with 4 technical replicates were undertaken (n=6). No assumptions were made about data distribution and all data are presented as median, interquartile/full range. Statistical analysis was undertaken using Analyse-it add in for Microsoft Excel (Analyse-it Software Ltd., Leeds, UK). Kruskal Wallis analysis of variance was used for each *in vitro* treatment, with Mann Whitney *post hoc* testing and correction for multiple comparisons as appropriate. For *in vivo* studies 5-9 rats per group were used. Data are presented as mean and standard deviation (SD) and were analysed using two-way repeated measures ANOVA with Bonferroni-corrected *post hoc* comparisons (Graph-Pad Prism v5.0, California, USA). A p value of <0.05 was considered to be significant.

Results

In vitro studies

Acid phosphatase activity

There was a significant dose dependent effect of paclitaxel exposure on DRG cell viability such that at 100uM paclitaxel the median [range] viability was 66.3 [58.0-88.3]%. However there was no additional effect of MitoVitE or Trolox (see Supplementary data). All subsequent measures were corrected for median viable cell number.

Mitochondrial function

Exposure of cells to paclitaxel at all doses without antioxidants resulted in decreased JC-1 red/green fluorescence ratio, indicating a loss of mitochondrial membrane potential ($p<0.0001$, Fig 1A). Mitochondrial membrane potential in cells without paclitaxel was similar to cells with antioxidant only, but in cells co-treated with paclitaxel and MitoVitE, membrane potential was only decreased at the highest dose of paclitaxel (Fig 1A). Cells treated with Trolox had similar loss of membrane potential to paclitaxel alone (Fig 1A), suggesting that it was ineffective at preventing loss of potential induced by paclitaxel. Mitochondrial metabolic activity was significantly lower in cells treated with paclitaxel regardless of dose ($p<0.0001$, Fig 1B). In cells co-treated with MitoVitE there was no decrease in metabolic activity even at the highest paclitaxel dose (Fig 1B). Trolox in contrast, worsened the loss of metabolic activity (Fig 1B).

Glutathione (GSH)

Compared to control treatments, cellular GSH levels were significantly lower in cells exposed to paclitaxel without antioxidants, independent of the paclitaxel dose ($p<0.0001$, Fig 1C). Co-exposure of paclitaxel with MitoVitE but not Trolox, mitigated the effect on GSH (Fig 1C).

Cancer cell cytotoxicity

Exposure of the A2780 ovarian carcinoma cell line or the MCF7 breast adenoma cell line to paclitaxel resulted in dose-dependent decreases in acid phosphatase activity, indicating loss of cell viability, such that at 100µM paclitaxel, only 60% of cells were viable. Co-exposure of cells to paclitaxel plus MitoVitE showed similar loss of viability (Figs 2A and 2B). Similar results were seen in Trolox treated ovarian cancer cells (Fig 2A), but paclitaxel mediated killing of breast cancer cells was less effective in the presence of Trolox (Fig 2B).

As a result of the promising effects of MitoVitE observed *in vitro* we then went on to assess its effects *in vivo*, with duloxetine as comparator.

In vivo studies

None of the treatments had an effect on weight gain (Supplementary file). There was also no effect of MitoVitE or duloxetine on withdrawal thresholds in the absence of paclitaxel administration, i.e. in naïve animals (see Supplementary file).

Mechanical withdrawal thresholds of a hind paw were examined, every second day, at 3, 6, and 9h after the daily injection of MitoVitE or duloxetine (Fig 3). Rats receiving paclitaxel with vehicle control treatments had significantly lower withdrawal threshold values compared to those receiving Cremophor from 4 days following the first paclitaxel injection until the end of the study (day 14). Rats receiving paclitaxel plus duloxetine had significantly higher withdrawal threshold values than rats given paclitaxel plus vehicle control on days 8-12. However rats receiving paclitaxel plus MitoVitE had significantly higher withdrawal threshold values than rats given paclitaxel plus vehicle control, over the longer timeframe of days 4-14 (with the exception of day 6).

For both MitoVitE and duloxetine treatment groups, significantly increased withdrawal thresholds were not consistent across the 3, 6 and 9h time points indicating time-dependent drug effects (Fig 3). To directly assess this, area under the curve (AUC) values between days 0 and 14 were calculated for each individual animal at each time point (Fig 4A). There was no significant difference between 3, 6 and 9h time points in the rats receiving either Cremophor or paclitaxel plus vehicle control. However in rats receiving paclitaxel plus duloxetine there was a significant effect of time, with a peak effect at 6h. In contrast, although there was a significant effect of time in the rats receiving paclitaxel plus MitoVitE there was a progressively increasing effect between 3 and 9h. This led us to assess an additional 24hr time point following the last drug/vehicle administration (day 14, Fig 4B). There was a significant effect at the 6h time point only in rats treated with paclitaxel plus duloxetine, compared with rats given paclitaxel plus vehicle control. The effect was more prolonged in rats receiving paclitaxel plus MitoVitE with a significant effect at 6, 9 and 24h (Fig 4B).

Discussion

We found that in DRG cells *in vitro*, paclitaxel affected aspects of mitochondrial function. There was loss of mitochondrial membrane potential, reduced metabolic activity, and loss of glutathione. Co-treatment of cells with paclitaxel plus MitoVitE but not Trolox, protected against these effects. MitoVitE also did not decrease the ability of paclitaxel treatment to kill cancer cells in two different cancer cell lines, whilst cytotoxicity against breast cancer cells was decreased by Trolox. In a rat model of chemotherapy induced neuropathic pain, we also showed that MitoVitE decreased paclitaxel-induced mechanical hypersensitivity to a similar degree as duloxetine. Moreover, at the doses tested, the duration of action of MitoVitE was more prolonged than that of duloxetine.

Chemotherapy remains the mainstay of cancer treatment for solid tumours but side effects can be severe enough to limit treatment. Paclitaxel acts by binding to microtubules and causing arrest of mitosis in cancer cells, followed by apoptotic cell death. Although proliferating cancer cells are susceptible to the action of paclitaxel, neuronal cells are also targets for damage and it has been shown that paclitaxel initiates opening of the mitochondrial permeability transition pore leading to loss of mitochondrial function in DRG cells.²¹ In paclitaxel treated rats, neuropathic pain behaviour was shown previously to be associated with evidence of mitochondrial damage in peripheral nerves,⁹ and administration of mitochondrial poisons such as rotenone was seen to worsen such behaviours.²²

The main function of mitochondria is to produce energy via oxidative phosphorylation but they are also an important source of ROS production, essential for signalling pathways. Although excessive mitochondrial ROS can be detrimental, antioxidant systems work in synergy to control potential damage under normal conditions.⁵ Exposure of a variety of cell types to paclitaxel has been shown to result in increased ROS production and oxidative stress^{7 8} and in animals, treatment with spin trap global radical scavengers reduced pain behaviour induced by paclitaxel.²³ Recently, an inhibitor of mitochondrial p53 accumulation was reported to limit paclitaxel induced mitochondrial damage and prevented mechanical allodynia, supporting the mechanistic importance of mitochondrial dysfunction and the rationale for targeting treatment specifically at mitochondria.²⁴

Antioxidants can be modified to specifically target mitochondria and may be more effective than those that do not. MitoVitE is able to enter mitochondria by virtue of the TPP cationic conjugate which enables accumulation several hundred fold inside the mitochondrion relative to cytosolic levels. It has been shown to be many more times more effective at protecting against oxidative damage to mitochondria than other non-targeted antioxidants^{10 11} and we and others have shown it to be effective in other disease models involving mitochondrial

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3 damage and oxidative stress.^{25 26} Trolox is a water soluble derivative of α -tocopherol which
4 permeates cells easily but cannot protect inside mitochondria. It has been shown to be
5 beneficial against oxidative stress in both cells and animals but has far lower antioxidant
6 activity than MitoVitE in standard *in vitro* rat brain homogenate lipid peroxidation assays.²⁷ In
7 DRG cells *in vitro*, we found that MitoVitE but not Trolox, protected against mitochondrial
8 dysfunction induced by paclitaxel.
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12 Mitochondrial membrane potential is an indicator of the efficiency of the electron transport
13 chain which creates the membrane potential, and the inner membrane permeability.²⁸ Using
14 a novel flow cytometry technique, Zhang and colleagues reported that paclitaxel dose
15 dependently reduced mitochondrial membrane potential of isolated mitochondria from HeLa
16 cells.²⁹ JC-1 is a widely used tool for measurement of mitochondrial membrane potential and
17 is thought to be a more reliable measure than other cationic probes^{16 28} although it must be
18 interpreted alongside other measures of mitochondrial function. In isolated mitochondria
19 from human neuroblastoma, paclitaxel induced release of cytochrome C and loss of
20 mitochondrial membrane potential³⁰ and in human melanoma cell lines paclitaxel exposure
21 resulted in decreased expression of mitochondrial uncoupling protein 2 (UCP2) with increased
22 ROS generation and loss of mitochondrial membrane potential, suggesting opening of the
23 mitochondrial pore.³¹ We also found that paclitaxel caused loss of mitochondrial membrane
24 potential in intact DRG cells.
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33 We used AlamarBlue as a measure of mitochondrial metabolic activity; this compound is
34 catalysed by oxidoreductases in mitochondria and can be used as a global measure of
35 mitochondrial metabolic activity.^{16 17} AlamarBlue can be reduced by NADPH, FADH, FMNH,
36 NADH and the cytochromes thus allowing the respiratory chain to function to near
37 completion. We found that paclitaxel caused lower metabolic activity, and MitoVitE but not
38 Trolox, abrogated this. Decreased metabolic activity seen here in viable cells is probably an
39 adaptive mechanism as a result of increased UCP2 expression in response to increased ROS
40 formation. UCP2 acts as a uncoupler to reduce ROS formation and not ATP formation as
41 indicated by the degree of decline in the mitochondrial membrane potential. Decreased
42 metabolic activity represents a protective response.
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49 Increased total glutathione can suggest oxidative stress, but consumption and loss of GSH
50 can represent overwhelming of the glutathione system. Glutathione levels decreased in DRG
51 cells exposed to paclitaxel in the present study and this was less marked in cells treated with
52 MitoVitE but not Trolox. In mouse tissue slices, paclitaxel-mediated release of calcitonin
53 gene-related peptide, a sensory neuropeptide implicated in paclitaxel induced neuropathy,
54 was abolished by treatment with glutathione.³²
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Antioxidants are known to protect against chemotherapy-induced oxidative stress and so addition of antioxidants to cancer chemotherapeutic regimens could in theory decrease their efficacy in killing cancer cells. The efficacy of cell killing in breast carcinoma cell lines by some chemotherapeutic drugs was shown previously to be reduced by concomitant treatment with non-biological antioxidants.³³ Another study reported that although resveratrol, a naturally occurring antioxidant found in grapes and other red berries, reduced the efficacy of paclitaxel to kills some breast cancer cell lines, susceptibility of MCF-7 cells was not diminished.³⁴ However a subsequent study found increased cytotoxicity of paclitaxel in the presence of resveratrol even in paclitaxel resistant breast cancer cell lines.³⁵ However we found that MitoVitE did not inhibit the cytotoxic action of paclitaxel against two different types of cancer cells whereas Trolox seemed to reduce killing in the MCF7 cells. In attempts to improve drug delivery, vitamin E has been used to form D- α -tocopherol polyethylene glycol (PEG) 1000 succinate by the esterification of tocopherol succinate with PEG 1000, to create a redox sensitive paclitaxel pro-drug which apparently increases paclitaxel induced cytotoxicity in A549 cells.³⁶

We used the well-characterized rat model of paclitaxel-induced neuropathic pain, where rats exhibit reduced mechanical withdrawal thresholds as assessed using Von Frey filaments, indicative of mechanical allodynia.^{19 20} Several rat models of paclitaxel-induced CIPN exist, employing a wide range of doses, treatment regimens and routes of administration. The specific model used here is amongst the most widely used and best characterized of paclitaxel induced CIPN models.¹⁹ We found, at the doses tested, treatment with MitoVitE (a mitochondria targeted form of tocopherol) limited paclitaxel-induced mechanical hypersensitivity to a similar level as that seen with duloxetine and moreover had a longer duration of action. Duloxetine has been shown to be effective in reducing neuropathic pain in clinical trials in patients treated with chemotherapy.^{3 4} In rat models of neuropathy other than that induced by paclitaxel, tocopherol (naturally occurring lipid soluble vitamin E) was reported to reduce allodynia after sciatic nerve crush injury,³⁷ chronic constriction induced ischaemic injury³⁸ and oxaliplatin induced neuropathy.³⁹ MitoVitE has been shown to be more effective in reducing mitochondrial damage in rat models of sepsis, another condition involving oxidative stress^{25 26} but there have been no studies describing its use in neuropathy or pain.

MitoVitE has not been through Phase I trials and so cannot currently be used in patients; however this study demonstrates proof of concept regarding a beneficial effect of mitochondria targeted antioxidant protection for CIPN. Other antioxidants which specifically protect inside mitochondria may be suitable for clinical use.⁴⁰ Further work should assess different antioxidant doses, varied timings of administration in relation to pain onset and any

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3 potential synergy with duloxetine. It would also be of interest to study the impact on
4 underlying neuropathology by quantifying intraepidermal nerve fibre density.⁴¹
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6 In summary we showed that paclitaxel induced mitochondrial damage in dorsal root ganglion
7 cells *in vitro* and that this was ameliorated by MitoVitE but not Trolox. In a rat model of
8 paclitaxel induced neuropathic pain, MitoVitE was as effective at reducing mechanical
9 hypersensitivity as duloxetine, the first line treatment for patients with paclitaxel induced
10 neuropathic pain. These results confirm the role of mitochondrial oxidative damage in
11 paclitaxel-mediated CIPN and suggest novel future treatment strategies.
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27 **Declarations of interest**

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42 **Author contributions**

43 All authors made a substantial contribution to the conception and design, acquisition of data,
44 or analysis and interpretation of data, were involved in drafting the article or revising it
45 critically for important intellectual content and approved the final version. They have all
46 agreed to be accountable for all aspects of the work in terms of accuracy and integrity.
47
48
49

50 BM: conducted *in vitro* and *in vivo* experimental work, analysed *in vivo* data and contributed
51 to writing the paper.
52
53

54 CT: designed and supervised *in vivo* work and contributed to writing the paper.
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56 DL: helped conduct and supervised *in vitro* experimental work, and contributed to writing the
57 paper.
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LC: designed study, supervised conduct, and contributed to writing the paper.

HFG: conceived of/designed study, analysed data, supervised conduct and wrote the paper.

All authors contributed to and approved the final version of the paper.

For Peer Review

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Review

Legends to figures

Figure 1

Effect of a range of concentrations of paclitaxel plus 1 μ M MitoVitE or Trolox, or vehicle control on **A.** mitochondrial membrane potential, **B.** mitochondrial metabolic activity and **C.** glutathione (GSH) in dorsal root ganglion cells.

Results are presented as percentage of data at baseline i.e. vehicle control treated cells without paclitaxel but with antioxidant treatment, to facilitate comparisons between treatments. Data are shown as box and whisker plots showing median, interquartile and full range (n=6). P value is Kruskal Wallis. * = significantly different to without paclitaxel.

Figure 2

Effect of a range of concentrations of paclitaxel plus 1 μ M MitoVitE or Trolox, or vehicle control on cell viability in **A.** the A2780 ovarian carcinoma cell line and **B.** in the MCF-7 breast adenocarcinoma-like oestrogen-sensitive cell line.

Results are presented as percentage of data at baseline i.e. vehicle control treated cells without paclitaxel but with antioxidant treatment, to facilitate comparisons between treatments. Data are shown as box and whisker plots showing median, interquartile and full range (n=6). P value is Kruskal Wallis. * = significantly different to without paclitaxel.

Figure 3

Mechanical hind paw withdrawal thresholds of groups receiving Cremophor (black), paclitaxel with vehicle control (red), paclitaxel with duloxetine (green) and paclitaxel with MitoVitE (blue). Mechanical withdrawal thresholds measured at **A.** 3 hours **B.** 6 hours and **C.** 9 hours after drug or vehicle administration.

Data are shown as mean (SD), n=5-9 per treatment group. Two-Way ANOVA followed by Bonferroni *post hoc* test used to compare all groups to paclitaxel with vehicle control. o = Cremophor p<0.001, * = paclitaxel with MitoVitE p<0.05, + = paclitaxel with duloxetine p<0.05.

Figure 4

A. Area under the curve values of mechanical withdrawal thresholds at 3, 6 and 9h after drug/vehicle administration as displayed in Fig 3. Data are shown as mean (SD), n=5-9 per

1
2
3 treatment group. Two-way repeated measures ANOVA with Bonferroni-corrected post hoc
4 tests indicated time-dependent effects of both MitoVitE and duloxetine administration. **B.**
5 Mechanical withdrawal thresholds in the 24h following the last drug/vehicle administration
6 (day 14). Cremophor (black), paclitaxel with vehicle control (red), paclitaxel with duloxetine
7 (green) and paclitaxel with MitoVitE (blue).
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11 Two-Way ANOVA followed by Bonferroni *post hoc* test used to compare all groups to
12 paclitaxel with vehicle control. o = Cremophor $p < 0.001$, * = paclitaxel with MitoVitE $p < 0.05$,
13 + = paclitaxel with duloxetine $p < 0.05$.
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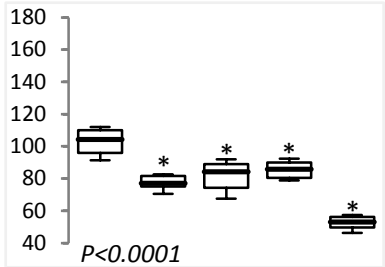
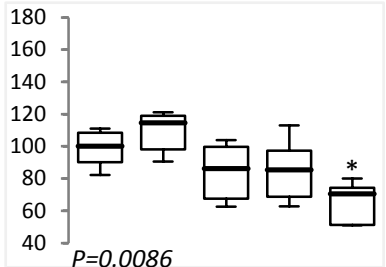
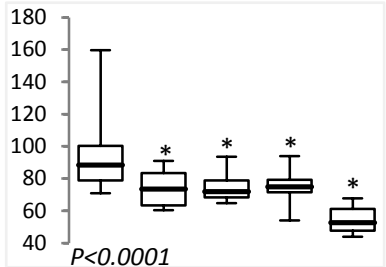
Fig 1

A

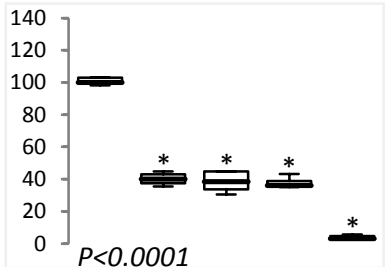
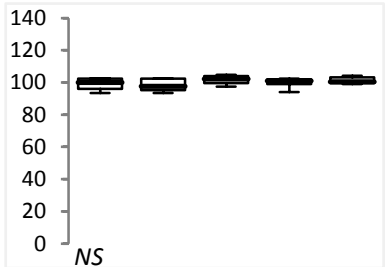
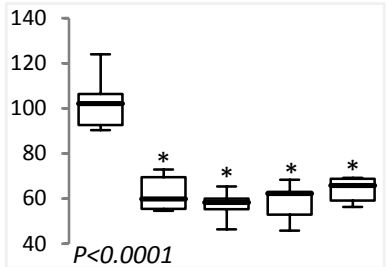
Vehicle control

MitoVitE

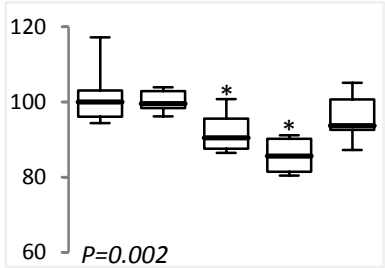
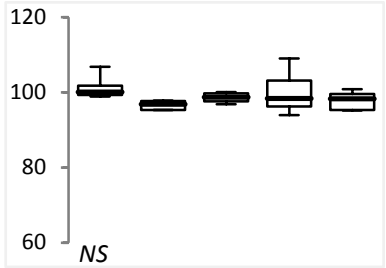
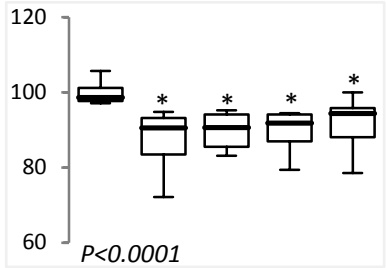
Trolox



B



C

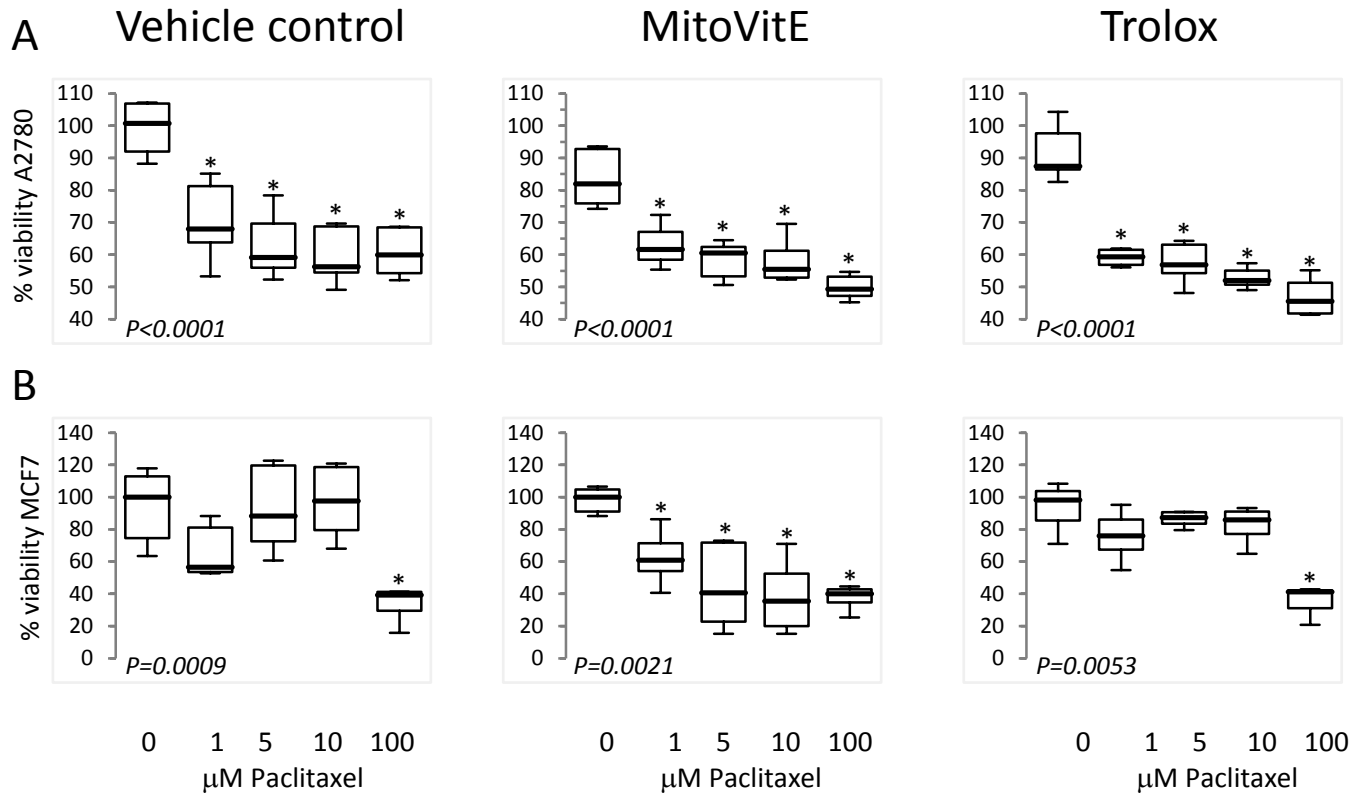


0 1 5 10 100
 μM Paclitaxel

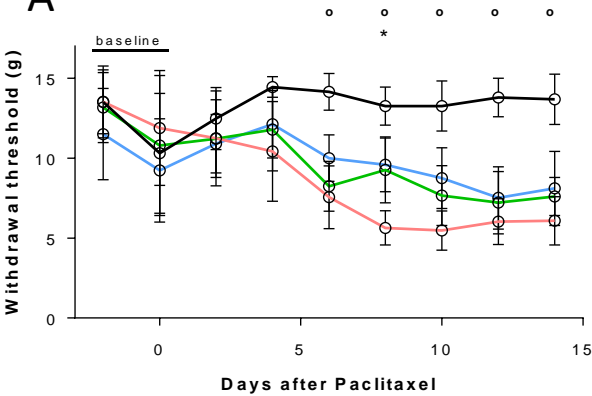
0 1 5 10 100
 μM Paclitaxel

0 1 5 10 100
 μM Paclitaxel

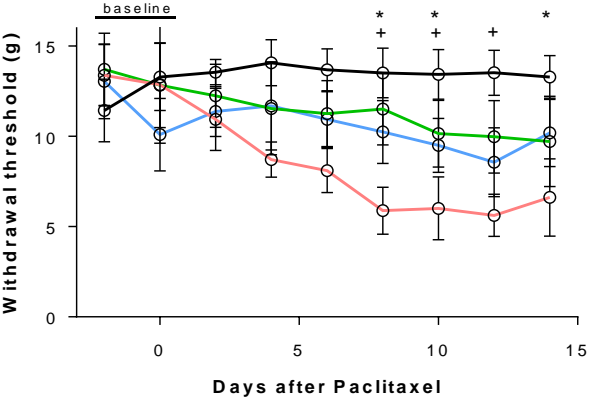
Fig 2



A



B



C

